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Epigenetic age estimation in a long-lived, deepwater scorpionfish: insights into epigenetic clock development

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Abstract: Age estimates are essential for fisheries assessment and management, but deepwater (>200 m) fishes are often difficult to age using traditional techniques. Therefore, age-predictive epigenetic clocks were developed for a model deepwater reef fish, blackbelly rosefish *Helicolenus dactylopterus*, using two tissue types (fin clips and muscle; n = 61 individuals; 9–60 years) and $\Delta^{14}C$-validated consensus age estimates. The influence of biological information (length and sex) on epigenetic clock accuracy, and the potential for developing a multi-tissue clock, were also assessed. Bisulfite-converted restriction site-associated DNA sequencing (bsRADseq) was used to identify CpG sites (cytosines followed by guanines) exhibiting age-correlated DNA methylation, and epigenetic clocks showed strong agreement ($R^2 > 0.98$) between predicted and consensus ages. Including length and sex data enhanced accuracy and precision ($R^2 > 0.99$; MAE < 1 year). Age-associated CpG sites were identified across tissues, but a multi-tissue clock performed poorly relative to single-tissue clocks. Overall, results demonstrate that accurate and precise epigenetic clocks can be developed for deepwater fishes, and the inclusion of biological information may enhance clock accuracy and precision.

Keywords: ageing, DNA methylation, multi-tissue, non-invasive, fish

Introduction

Age estimation provides fish life history information (e.g., age-at-length, age-at-maturity, age-related fecundity) that is essential for fisheries assessment and management, particularly when age-structured stock assessment models are used to estimate stock status (Ono et al. 2015). Techniques used for estimating age typically involve counting growth zones in a range of hard structures, including otoliths, vertebrae, scales, and fin rays (Campana 2001). Deepwater fishes, however, are often difficult to age using traditional techniques (e.g., otolith analysis), due largely to slow growth rates, long life spans, and the constancy of the environment in which they live, all of which can contribute to difficulty in discerning growth zones (annuli) and higher ageing error (Cailliet et al. 2001). Thus, the development of an alternative ageing approach would be of great utility.

While other alternative ageing approaches have been explored (e.g., Fourier transform near infrared spectroscopy, FT-NIRS; Helser et al. 2018; Passerotti et al. 2020), there is increasing interest in the development of DNA methylation-based, epigenetic ageing techniques (Piferrer and Anastasiadi 2023). This is due in large part to the fact that epigenetic ageing is inexpensive relative to otolith-based ageing, non-lethal, and well-suited for rapid age estimation (Mayne et al. 2023). DNA methylation is an epigenetic mechanism involving a chemical modification of the DNA, whereby the 5' carbon of cytosine is modified by the addition of a methyl group (CH_3; Moore et al. 2013). In vertebrates, DNA methylation typically occurs at cytosines that are followed by guanines, known as CpG sites, and has been implicated as an important mechanism for gene regulation (Bommarito and Fry 2019). Recent studies have demonstrated that changes in DNA methylation levels at certain CpG sites exhibit strong correlations with chronological age, leading to the development of age-predictive models based

on DNA methylation, referred to as epigenetic clocks (reviewed in Parrott and Bertucci 2019 and Piferrer and Anastasiadi 2023). Epigenetic clocks summarize age-associated increases (hypermethylation) or decreases (hypomethylation) in DNA methylation across a selected group of CpG sites throughout the genome, which can be used collectively to estimate chronological age (Piferrer and Anastasiadi 2023). While the utility of epigenetic age estimation has been demonstrated in several fish species (European sea bass *Dicentrarchus labrax*, Anastasiadi and Piferrer 2020; zebrafish *Danio rerio*, Mayne et al. 2020; Australian lungfish *Neoceratodus forsteri*, Murray cod *Maccullochella peelii*, and Mary River cod *Maccullochella mariensis*, Mayne et al. 2021a; Japanese medaka *Oryzias latipes*, Bertucci et al. 2021; northern red snapper *Lutjanus campechanus* and red grouper *Epinephelus morio*, Weber et al. 2022; and golden perch *Macquaria ambigua* spp., Mayne et al. 2023), the application of this ageing technique to a deepwater fish species has not yet been investigated. The development of epigenetic clocks in deepwater fishes would seem to be of great utility given the high error often associated with traditional ageing techniques for deepwater fishes (Cailliet et al. 2001; Campana 2005) and their vulnerability to overfishing given their longevity and slow growth (Devine et al. 2006).

Multi-tissue epigenetic clocks have been developed for mammals (e.g., humans, Horvath 2013; mice, Thompson et al. 2018, and pinnipeds, Robeck et al. 2023), but previously developed piscine epigenetic clocks have only involved DNA methylation levels identified in a single tissue type (e.g., fin, Mayne et al. 2020, Mayne et al. 2021a, Weber et al. 2022; muscle, Anastasiadi and Piferrer 2020, Weber et al. 2022; and liver, Bertucci et al. 2021). While tissue-specific patterns of DNA methylation have been identified in fishes (Venney et al. 2016; Zupkovitz et al. 2021), the presence of CpG sites exhibiting age-correlated methylation across tissue types (e.g., fin clips, muscle), and the potential development of multi-tissue, piscine epigenetic clocks, has

not been explored. Fin clips and muscle tissue are commonly sampled during fisheries research, thus the ability to use a single epigenetic clock for both tissue types may increase the ease with which age estimates could be obtained, and would also have important implications for future epigenetic clock development. In addition, the influence of incorporating biological information (e.g., length and sex) on the development and accuracy of piscine epigenetic clocks remains unknown (Piferrer and Anastasiadi 2023). Given that length data are often used as a proxy for age, and that epigenetic regulation plays a major role in sex determination in fishes (Yamagishi et al. 2022), it is possible that the inclusion of such biological information could enhance epigenetic clock accuracy and precision.

In this study, epigenetic clocks were developed for the blackbelly rosefish (*Helicolenus dactylopterus*), a demersal deepwater scorpionfish that inhabits soft bottom along the continental shelf and upper slope between 150 and 600 m (Mendonca et al. 2006). The blackbelly rosefish is widely distributed in the Atlantic Ocean and adjacent seas, including the northern Gulf of Mexico and Mediterranean Sea, and is commercially targeted in the northeastern Atlantic (Sequeira et al. 2015). Similar to many other deepwater fishes, blackbelly rosefish exhibit slow growth ($k = 0.08$ y^{-1}) and a long life span (maximum longevity >90 years), which implies low resilience to fishing pressure (Chamberlin et al. 2023). In addition, like many other deepwater fishes, blackbelly rosefish are difficult to age by counting growth zones in otoliths. Chamberlin et al. (2023) recently applied the bomb ^{14}C chronometer to blackbelly rosefish eye lens cores (i.e., age-0 metabolic C Δ^{14}C signatures) and validated otolith opaque zone counts (ages 9 to 90 years) as being accurate, overall. However, there was imprecision in ageing between paired otolith readers. The authors reported an index of average percent error (iAPE) of 4.48% for their

study, but 17.50% of their samples were estimated to be >50 years old and the presence of older ages often depresses iAPE.

Study objectives were to: (1) investigate the possibility of developing epigenetic clocks for the blackbelly rosefish using $\Delta^{14}C$-validated consensus ages, through the *de novo* identification of CpG sites exhibiting age-correlated DNA methylation; (2) assess the influence of biological information (i.e., length and sex) on the accuracy of epigenetic clocks; and (3) assess for the potential development of a multi-tissue epigenetic clock, through the identification of age-associated CpG sites that are shared across tissue types (fin clips and muscle). The accuracy and precision of the epigenetic clocks developed were compared to otolith-based ageing reported by Chamberlin et al. (2023) for the same species.

Methods

Otoliths and tissue samples were collected from blackbelly rosefish caught between 2020 and 2022 in the northcentral Gulf of Mexico with hook-and-line at depths of 338 to 519 m. Fish were handled in accordance with the Guide for the Care and Use of Laboratory Animals under protocols approved by the University of Florida Institutional Animal Care and Use Committee (Protocol #202111559). Captured fish were measured to the nearest mm fork length and total length. Otoliths were extracted from fish, rinsed with deionized water, and stored dry in paper coin envelopes. Eye balls were dissected, placed in plastic bags, and frozen at –20 °C. Approximately 4 g of white muscle tissue was dissected from the lateral musculature just below and in front of the dorsal fin, placed in a plastic bag, and frozen at –20 °C. Approximately 1 cm^2 pectoral fin clip samples were removed with scissors, immersed in 20% DMSO-0.25 M EDTA NaCl-saturated buffer (Seutin et al. 1991), and stored at room temperature until DNA extraction.

Fish age was estimated via opaque zone counts in otolith thin sections by two independent readers (WFP and DWC), with ageing data originally reported in Chamberlin et al. (2023). The index of average percent error (iAPE) was computed between readers for the subset of the Chamberlin et al. (2023) samples that were included in epigenetic clock development:

$$iAPE = \frac{1}{n}\sum_{j=1}^{n}\left[\frac{1}{R}\sum_{i=1}^{R}\frac{|X_{ij} - X_j|}{\overline{X}_j}\right] x\ 100 \qquad \text{(Equation 1)}$$

where n = number of samples aged; R = number of age estimates per sample; X_{ij} is the age estimate for the i^{th} reader's age estimate for the j^{th} fish ; and $\overline{X}_j$ is the mean age estimate calculated for the j^{th} fish (Beamish and Fournier 1981). In addition, right eye lenses were cored following freeze drying and analyzed for $\Delta^{14}C$, as reported in Chamberlin et al. (2023). Prior to epigenetic clock development, consensus age for each sample was developed by WFP and DWC after comparison of reader-estimated ages and $\Delta^{14}C$-predicted ages relative to the Bayesian spline model fitted by Chamberlin et al. (2023).

Genomic DNA was extracted from fin clip and muscle tissue samples using the Mag–Bind Blood & Tissue DNA Kit (Omega Bio-tek, Inc., Norcross, USA), and a library was prepared for bisulfite-converted restriction site-associated DNA sequencing (bsRADseq), following a modified version of the Trucchi et al. (2016) protocol (described in Weber et al. 2022). Briefly, restriction digests were performed using *Mfe*I-HF, and unique hemi-methylated barcoded adaptors were ligated to each sample (Peterson et al. 2012). Samples were then pooled, sheared (Covaris M220 Ultrasonicator, Covaris, Inc., Woburn, USA), and size selected using a Pippin Prep Size Selection System (Sage Science, Inc., Beverly, USA). The library was then split into two portions, and one portion was bisulfite-treated using an EpiTect Plus Bisulfite Kit (Qiagen, Hilden, Germany). Bisulfite treatment converts unmethylated cytosines into uracils through chemical deamination, and uracils are subsequently replaced by thymines during PCR

amplification. This results in predictable base substitutions at all unmethylated cytosines, which can be identified by comparing sequences from the treated portion to the untreated portion (Trucchi et al. 2016). The library (including both the treated and untreated portion) was sequenced across a single lane on an Illumina NovaSeq 6000 (Illumina, Inc., San Diego, USA). Bisulfite-treated reads were mapped to a reference genome constructed from the untreated reads using the *dDocent* pipeline (Puritz et al. 2014) with c = 0.88, K_1 = 2, and K_2 = 1. Mapped reads were then filtered to retain primary alignments, proper pairs, and those with a mapping quality ≥40.

CpG sites that could not be successfully genotyped in the untreated portion or that were identified as potential single nucleotide polymorphisms (SNPs; defined as sites where >5% of total untreated reads across individuals displayed a cytosine to thymine substitution on the forward strand or guanine to adenine substitution on the reverse strand) were removed from the dataset. CpG sites were then filtered to retain only sites present in ≥80% of individuals per tissue type. Percent methylation was estimated as the number of methylated reads divided by the total number of reads, and per-site 95% confidence intervals were calculated around the estimate for each individual (Clopper and Pearson 1934). Only those sites with confidence intervals <0.75 in at least 80% of individuals were retained, which is roughly equivalent to an average of 30 reads per site (Weber et al. 2022).

To identify all CpG sites exhibiting age-correlated DNA methylation, a Bayesian framework was used to estimate the parameters of a generalized linear model (GLM) that included consensus age, tissue type, and sex as fixed factors and individual as a random factor, using the package *rstanarm* version 2.19.3 (Goodrich et al. 2020). The response variable was the binomial expression of the number of methylated reads (n) and the total number of reads for each

sample (k) at a given CpG site (n/k). GLMs were considered to have converged if the effective sample size (n_eff), was greater than 2,000 and the Gelman-Rubin convergence diagnostic was less than 1.01 (Lunn et al. 2013; Muth et al. 2018). CpG sites with a 95% credible interval that did not include zero for the slope of consensus age versus methylation were considered to exhibit significant age-correlated methylation. For each CpG site that exhibited age-correlated methylation, individuals with over-dispersed confidence intervals (>0.75) were entered as missing data. Because downstream analysis does not allow for missing data, methylation frequencies at missing individuals/sites were imputed using the package *mice* version 3.9.0 (van Buuren and Groothuis-Oudshoorn 2011).

The relationship between consensus age and percent methylation across CpG sites was characterized using elastic net penalized regression modeling, as implemented in the package *glmnet* version 4.0.2 (Friedman et al. 2010). Independent models were constructed for each tissue type (i.e., fin clips and muscle) and for both tissue types combined. Given the genomic approach used (i.e., bsRADseq), and the large number of age-correlated CpG sites identified, the number of CpG sites included in each of the three models must be reduced prior to penalized regression modeling. This was accomplished iteratively by evaluating independent Pearson correlation values (Bertucci et al. 2021), obtained using the 'corr.test' function from the package *psych* version 2.2.9, and model slope coefficients from 'cv.glmnet' run with an alpha (α) of 0. For each model, 80% of samples were randomly assigned to a training dataset and the remainder into a testing dataset, using the 'partition' function from the package *splitTools* version 0.3.2 (Mayer 2003). Final predictive models were then constructed using the training datasets and the 'cva.glmnet' function in *glmnet*, which simultaneously cross validates both the penalty and alpha parameters. Penalized regression modeling (including CpG site reduction) was then repeated

with the simultaneous inclusion of fork length and total length, and then performed separately for males and females. Finally, to determine if an epigenetic clock developed using one tissue type (e.g., fin clips) could be used to obtain accurate age estimates from another tissue type (e.g., muscle), the CpG sites included in the final fin clip model were used to predict age using the muscle tissue samples, and vice-versa.

Model performance for training and testing datasets was assessed using linear regressions and by comparing mean absolute error (MAE) rates between consensus ages and ages predicted by the epigenetic clocks. To assess for potential overfitting, Fisher's *F*-tests ($\alpha = 0.05$), conducted using the 'var.test' function from the package *stats* version 3.6.0, were used to determine if the variance in the residuals differed between training and testing datasets. Relative error was calculated by dividing the absolute error by the consensus age, and linear regressions were conducted on consensus age versus relative error in the testing datasets, to determine if error in the models increased with increasing age. Lastly, iAPE was computed between consensus ages and predicted ages for all epigenetic clocks (Equation 1). All analyses were conducted in *R* version 3.6.0 (R Core Team 2023).

Results

Sampling and age estimation

In total, 61 blackbelly rosefish were sampled for otoliths and tissues, with fin clip and muscle tissue samples available for 32 individuals, fin clips only from 24 individuals, and muscle tissue only from 5 individuals. Otolith sections (Fig. 1) were produced for these samples and paired age estimates (readers: WFP and DWC) analyzed and reported in Chamberlin et al. (2023). The overall index of average percent error (iAPE) between readers reported for

blackbelly rosefish (n = 356) aged in Chamberlin et al. (2023) was 4.48% for fish that ranged in age from 9 to 90 years. Recomputing the iAPE for the subset of samples (n = 61) analyzed herein for epigenetic clock development produced an estimate of 6.47%. Consensus ages among these samples ranged from 9 to 60 years (Supp. Data[1]).

Epigenetic clock development

Across all individuals (n = 61) and both tissue types, 2,959,164 CpG sites were recovered. A total of 175,973 sites either could not be successfully genotyped in the untreated portion of the library or were identified as potential SNPs and removed from the dataset.

Across all fin clips (n = 56), 156,529 CpG sites had sufficiently tight confidence intervals in at least 80% of individuals. Mean (± SD) global CpG methylation was 85.20% (± 0.91%). Bayesian GLMs identified 10,139 CpG sites that exhibited significant age-correlated methylation, and penalized regression analysis retained 316 CpG sites in the age-predictive model. Strong agreement was observed between consensus age and predicted age in both the training (R^2 = 0.98) and testing (R^2 = 0.98) datasets (Fig. 2A,B; Table 1). Mean absolute error (MAE) was 1.66 and 1.62 years in the training and testing datasets, respectively (Fig. 2A,B; Table 1). Variance in the residuals did not differ between training and testing datasets (Fisher's *F*-test, p = 0.81), and relative error in the testing dataset did not increase with increasing age (p = 0.35; R^2 = 0.09).

Across all muscle tissue samples (n = 37), 115,569 CpG sites had sufficiently tight confidence intervals in at least 80% of individuals. Mean (± SD) global CpG methylation was 85.40% (± 0.45%). Bayesian GLMs identified 5,886 CpG sites that exhibited significant age-

[1]Supplementary data are available with the article at XXX.

237 correlated methylation, and penalized regression analysis retained 312 CpG sites in the age-
238 predictive model. As above for the fin clip samples, strong agreement was observed between
239 consensus age and predicted age in both the training (R^2 = 0.99) and testing (R^2 = 0.98) datasets
240 (Fig. 2C,D; Table 1). MAE was 2.07 and 2.14 years in the training and testing datasets,
241 respectively (Fig. 2C,D; Table 1). Variance in the residuals did not differ between training and
242 testing datasets (Fisher's *F*-test, p = 0.70), and relative error in the testing dataset did not
243 increase with increasing age (p = 0.66; R^2 = 0.03).
244
245 *Influence of biological information*
246 The inclusion of length data (fork length and total length) enhanced the accuracy and
247 precision of the fin clip clock (R^2 = 0.99 and MAE = 0.90 years in the testing dataset; Fig. 3B;
248 Table 2) and the muscle tissue clock (R^2 = 0.99 and MAE = 1.25 years in the testing dataset; Fig.
249 3D; Table 2). Including sex data and performing the same analysis separately for males and
250 females further enhanced the accuracy and precision of both the fin clip clock (R^2 = 0.99, MAE =
251 0.43 years for males and R^2 = 0.99, MAE = 0.42 years for females in the testing dataset; Fig. 4B;
252 Table 2) and the muscle tissue clock (R^2 = 0.99, MAE = 0.78 years for males and R^2 = 0.99,
253 MAE = 0.80 years for females in the testing dataset; Fig. 4D; Table 2).
254
255 *Multi-tissue epigenetic clock development*
256 Across both tissue types (n = 56 fin clips; n = 37 muscle tissue), 129,916 CpG sites had
257 sufficiently tight confidence intervals in at least 80% of individuals. Mean (± SD) global CpG
258 methylation was 85.20% (± 0.78%). Bayesian GLMs identified 5,063 CpG sites that exhibited
259 significant age-correlated methylation, and no significant difference in methylation between

tissue types. For modeling purposes, however, all 15,953 CpG sites that exhibited a significant relationship with age, regardless of significant relationships with tissue type or their interaction in the Bayesian GLM, were included. Penalized regression analysis retained 498 CpG sites in the age-predictive model. The multi-tissue clock performed relatively well for the fin clip samples (R^2 = 0.94 and MAE = 3.08 years in the testing dataset; Fig 5B; Table 1), but not for the muscle tissue samples (R^2 = 0.77 and MAE = 5.43 years in the testing dataset; Fig. 5B; Table 1). Variance in the residuals did not differ between the training and testing datasets for either tissue type (Fisher's *F*-tests; fin clips, p = 0.83; muscle tissue, p = 0.58). Forty-four of the 498 CpG sites included in the multi-tissue clock were also included in the fin clip clock, and 21 of the 498 CpG sites included in the multi-tissue clock were included in the muscle tissue clock.

The epigenetic clock developed using the fin clip samples (n = 316 CpG sites) was unable to accurately predict the ages of the muscle tissue samples (R^2 = 0.10, MAE = 13.30 years; Fig. S1A). Similarly, the epigenetic clock developed using the muscle tissue samples (n = 312 CpG sites) was unable to accurately predict the ages of the fin clip samples (R^2 = 0.18, MAE = 12.19 years; Fig. S1B).

Epigenetic clock index of average percent error

The iAPE for epigenetic clocks developed using fin clips ranged from 1.04% to 2.77% for testing datasets and 2.95% to 4.46% for training datasets (Table 3). Among muscle tissue epigenetic clocks, iAPE ranged from 1.45% to 4.31% for testing datasets and 2.67% and 5.23% for training datasets (Table 3). The iAPE for all epigenetic clocks was lower than the iAPE computed between otolith readers (6.47%) for the individuals analyzed herein (n = 61).

Discussion

The development of accurate and precise epigenetic clocks for the blackbelly rosefish suggests epigenetic clocks could provide a non-lethal, efficient, and effective ageing approach for deepwater fishes that are difficult to age using traditional ageing techniques. The high accuracy and precision reported are particularly important in the context of fisheries assessment and management, given that error in age estimation propagates as bias or imprecision in the estimation of population parameters (e.g., growth, mortality, yield per recruit) and stock assessment outputs (Lai and Gunderson 1987). In addition, while the demand for fish ageing is increasing, otolith ageing is a time- and cost-intensive process, often limiting the amount of data that can be collected and incorporated into stock assessments (Helser et al. 2019). The results presented herein contribute to a growing body of literature demonstrating the potential for epigenetic ageing to greatly impact the way in which age estimates are generated and the volume of data available for stock assessments.

Single-tissue epigenetic clocks developed here were both accurate and precise, with greater levels of precision than reported for ageing of blackbelly rosefish using traditional annuli counts in otoliths (Chamberlin et al. 2023). While the epigenetic clock developed using the fin clips appeared to perform better than the clock developed using muscle tissue, this result is likely due in part to the smaller sample size for muscle tissue than fin clips (n = 37 and n = 56, respectively). The accuracy and precision of epigenetic clocks are likely attributable to a variety of factors, including the number of individuals incorporated, the age range of those individuals, the accuracy of the age estimates used to train the clock, the number of age-correlated CpG sites evaluated, and the degree of environmental influence on the epigenome of the species of interest. A recent simulation study indicated that a minimum sample size of 70 individuals in the training

dataset is necessary to maximize epigenetic clock accuracy and precision (Mayne et al. 2021b), and while that study could not account for all of the factors listed above, results of the present study are consistent with that guidance, as the clock developed with the larger training dataset (fin clip) performed better than the clock developed with the smaller training dataset (muscle tissue). Having said that, among the 51 tissues and cell types included in the human epigenetic clock, skeletal muscle tissue was among the poorer predictors of age (Horvath 2013). Therefore, a biological basis for the decreased accuracy observed in the muscle tissue clock (e.g., plasticity, high stem cell content) cannot be ruled out.

Notably, model performance did not decrease with increasing age in the fin clip or muscle tissue clocks, which is contrary to results from previous studies that utilized evolutionarily conserved age-associated DNA methylation at CpG sites identified in the zebrafish (*Danio rerio*, e.g., Mayne et al. 2021a; Mayne et al. 2023). This is particularly important for long-lived species like the blackbelly rosefish, and is likely the result of characterizing a large number of independent CpG sites exhibiting age-correlated methylation specifically within the blackbelly rosefish genome and across individuals exhibiting a wide age range. Moreover, the use of consensus otolith-derived age estimates, validated via application of the bomb radiocarbon chronometer (Chamberlin et al. 2023), to train the epigenetic clocks likely contributed to the observed accuracy and precision. Thus, when maximizing accuracy is the primary focus, it is important to consider the potential benefits of developing epigenetic clocks *de novo* in the species of interest.

The inclusion of biological information (i.e., length and sex data) enhanced the accuracy and precision of the fin clip and muscle tissue clocks. Length data can be easily and non-invasively collected and could thus be included in epigenetic-based age estimation with minimal

effort. The epigenetic clocks developed with length data also required fewer CpG sites to predict age, which is important to consider when generating assays for production ageing (i.e., the efficient generation of age estimates for large numbers of individuals needed for fisheries assessment; Passerotti et al. 2020), such as via genotyping-in-thousands by sequencing (GT-seq; Campbell et al. 2015), because PCR assays are easier to design with fewer loci. While Anastasiadi and Piferrer (2020) reported that the inclusion of length data did not have an effect on epigenetic age estimation in the European sea bass (*Dicentrarchus labrax*), this difference may be due to the fact that length data were treated as a covariate in a regression of predicted age versus chronological age in that study, while length data were treated as predictor variables during epigenetic clock development in the present study. The accuracy and precision of the epigenetic clocks further improved when males and females were modeled separately, suggesting patterns of age-associated DNA methylation are somewhat sex-specific. This is supported by previous research conducted on humans (reviewed in Yusipov et al. 2020) and roe deer (*Capreolus capreolus*, Lemaitre et al. 2022), where distinct patterns in epigenetic ageing were identified between sexes. Because many teleost species of commercial interest exhibit large differences in growth rate and maximum size between sexes (e.g., Atlantic cod *Gadus morhua* and Pacific halibut *Hippoglossus stenolepis*, Pifferer and Anastasiadi 2023), it is possible that the design of sex-specific epigenetic clocks will be necessary to maximize accuracy and precision. While sex data are routinely and non-invasively collected for species displaying sexual dimorphisms (e.g., elasmobranchs and some teleosts, including mahi mahi *Coryphaena hippurus* and black rockfish *Sebastes melanops*), sex data can also be non-invasively obtained via genetic methods (e.g., genotyping sex-specific markers; Bargen et al. 2015) for species with genetic sex

351 determination. Thus, the inclusion of sex-specific markers in epigenetic clock panels could allow
352 for the determination of both age and sex via the same tissue sample.

353 A number of CpG sites exhibiting age-correlated DNA methylation were shared between
354 fin clip and muscle tissue samples, allowing for the development of a multi-tissue piscine
355 epigenetic clock. However, the relatively poor performance of the multi-tissue clock, when
356 compared to the clocks built separately for fin clips and muscle tissue, suggests age-related
357 changes in DNA methylation may be tissue-specific. This is supported by the fact that no CpG
358 sites were shared between the fin clip and muscle tissue clocks developed herein, and further
359 supported by the fact that the epigenetic clocks developed using one tissue type (e.g., fin clips)
360 were unable to accurately predict the ages of the other tissue type (e.g., muscle tissue). This is
361 consistent with results from previous studies conducted on humans (Horvath 2013; Christensen
362 et al. 2009), rats (Thompson et al. 2010), mice (Thompson et al. 2018), and common bottlenose
363 dolphins (Robeck et al. 2021a), all of which demonstrated tissue-specific age-related changes in
364 DNA methylation. Thus, while the identification of age-associated CpG sites shared between
365 tissue types allowed for the development of a multi-tissue clock, the simultaneous development
366 of tissue-specific clocks likely requires similar time and effort, and the results presented herein
367 support the notion that single-tissue epigenetic clocks are generally more accurate than multi-
368 tissue clocks (Robeck et al. 2021b).

369 Age estimates for all muscle tissue samples from individuals >50 years old were
370 underpredicted in the multi-tissue clock, while all but one fin clip sample from individuals >50
371 years old were overpredicted, suggesting the presence of tissue-specific differences in 'tick rates'
372 (i.e., the rates of change in DNA methylation). While tissue-specific differences in tick rates
373 have not been documented in fishes (Piferrer and Anastasiadi 2023), this phenomenon is well

established in humans (Horvath and Raj 2018). The use of skeletal muscle tissue led to the underprediction of age in a multi-tissue epigenetic clock developed for humans, perhaps due to the rejuvenation of DNA methylation profiles in muscle tissue via myosatellite cells – muscle stem cells involved in muscle growth and regeneration following injury or disease (Horvath 2013). Recognizing that tissue-specific differences in tick rates may exist in fishes, caution should be taken against building epigenetic clocks with tissues prone to epigenetic age acceleration (i.e., higher tick rates; e.g., kidney, brain; Piferrer and Anastasiadi, 2023).

Overall, the present study adds to the growing body of literature on the development and application of epigenetic clocks for fishes (reviewed in Anastasiadi and Pifferer 2023 and Piferrer and Anastasiadi 2023). The results presented for blackbelly rosefish demonstrate that accurate and precise epigenetic clocks can be developed for long-lived, deepwater fish species, which are often vulnerable to overfishing (Devine et al. 2006) and difficult to age using traditional techniques (Campana 2005; Kastelle et al. 2020). In addition, this study demonstrates the inclusion of biological information (i.e., length and sex), which is commonly available and easily and nondestructively obtained, can improve clock performance. Finally, while the ability to develop a multi-tissue piscine clock was demonstrated herein, the results suggest age-related changes in DNA methylation are tissue-specific and single-tissue epigenetic clocks may provide higher accuracy than multi-tissue clocks. When compared to traditional ageing techniques (e.g., otolith analysis), the use of epigenetic clocks to obtain age estimates is relatively inexpensive (Mayne et al. 2021a), non-lethal, and not hindered by between-reader precision issues. Thus, while epigenetic clocks have the potential to greatly advance the generation of age estimates for fisheries research and management, continued research on the best practices to enhance clock accuracy and precision (e.g., using validated age estimates to train models, incorporating

biological information, the choice of tissue type, species-specific versus multi-species clocks, and more sophisticated modeling approaches) is warranted.

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Competing interests statement

The authors declare there are no competing interests.

Author contribution statement

DNW: conceptualization, data curation, methodology, formal analysis, writing – original draft; **ATF:** conceptualization, methodology, formal analysis, writing – review and editing; **DWC:** data curation, writing – review and editing; **WFP:** conceptualization, data curation, supervision, funding acquisition, writing – review and editing; **DSP:** conceptualization, methodology, resources, supervision, funding acquisition, writing – review and editing.

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Data availability statement

Datasets (raw and filtered) and data analysis scripts are available at https://github.com/marinegenomicslab/BBRF_EpigeneticAgeing. Raw bsRADseq sequences will be made publicly available at the conclusion of a separate ongoing study.

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Table 1. Summary statistics for epigenetic clocks developed for the blackbelly rosefish using fin clips, muscle, and both tissue types combined, including the age range of fish in years, the number of CpG sites included in the model, the number individuals included in training and testing datasets, the coefficient of determination (R^2), and the mean absolute error (MAE) reported in years.

Model	**Age range**	**CpG sites**	**Dataset**	**Individuals**	R^2	**MAE**
Fin clip	9–59	316	Training	44	0.98	1.66
			Testing	12	0.98	1.62
Muscle	9–60	312	Training	29	0.99	2.07
			Testing	8	0.98	2.14
Multi-tissue						
Fin clip	9–59	498	Training	44	0.93	2.94
			Testing	12	0.94	3.08
Muscle	9–60	498	Training	29	0.92	5.75
			Testing	8	0.77	5.43

Table 2. Summary statistics for epigenetic clocks developed with the inclusion of biological information (i.e., length and sex data), including the age range of fish in years, the number of CpG sites included in the model, the number individuals included in training and testing datasets, the coefficient of determination (R^2), and the mean absolute error (MAE) reported in years.

Model	Age range	CpG sites	Dataset	Individuals	R^2	MAE
Fin clip	9–59	316	Training	44	0.98	1.66
			Testing	12	0.98	1.62
Fin clip + length	9–59	313	Training	44	0.99	1.47
			Testing	11	0.99	0.90
Fin clip + length by sex						
Males	9–37	448	Training	23	0.99	0.93
			Testing	6	0.99	0.43
Females	11–59	306	Training	20	0.99	1.26
			Testing	5	0.99	0.42
Muscle	9–60	312	Training	29	0.99	2.07
			Testing	8	0.98	2.14
Muscle + length	9–60	250	Training	29	0.99	2.12
			Testing	8	0.99	1.25
Muscle + length by sex						
Males	9–60	685	Training	13	0.99	1.16
			Testing	3	0.99	0.78
Females	13–60	489	Training	16	0.99	0.94
			Testing	4	0.99	0.80

Table 3. Index of average percent error (iAPE) for blackbelly rosefish epigenetic clock predicted age estimates versus consensus age estimates produced from paired-reader ages and eye lens core $\Delta^{14}C$ values. Training dataset iAPE estimates were computed via leave-one-out, while test dataset iAPE values were produced from age predictions for samples not included in epigenetic clock development.

Model	Training n	Training iAPE %	Testing n	Testing iAPE %
Fin clip	44	4.46	12	2.77
Fin clip + length	44	3.86	11	2.09
Fin clip + length by sex	43	2.95	11	1.04
Muscle	29	5.23	8	4.31
Muscle + length	29	5.02	8	2.77
Muscle + length by sex	29	2.67	7	1.45

Figure 1. Digital image of a sagittal otolith section viewed with transmitted light from a 352 mm total length female blackbelly rosefish (N1307) with a consensus age estimate of 60 years.

Figure 2. Epigenetic age predictions versus consensus ages for epigenetic clocks developed using fin clip samples (A,B; red circles) and muscle tissue samples (C,D; blue circles). The lefthand panels depict the training datasets and righthand panels the testing datasets. Dashed lines indicate lines of 1:1 agreement between predicted and consensus ages. Solid lines represent linear regression fits to the data. The coefficient of determination (R^2) and mean absolute error (MAE) reported in years are displayed on panels.

Figure 3. Epigenetic age predictions versus consensus ages for epigenetic clocks developed using fin clip samples (A,B; red circles) and muscle tissue samples (C,D; blue circles) with the inclusion of length data. The lefthand panels depict the training datasets and righthand panels the testing datasets. Dashed lines indicate lines of 1:1 agreement between predicted and consensus ages. Solid lines represent linear regression fits to the data. The coefficient of determination (R^2) and mean absolute error (MAE) reported in years are displayed on panels.

Figure 4. Epigenetic age predictions versus consensus ages for epigenetic clocks developed using fin clip samples (A,B; red) and muscle tissue samples (C,D; blue) with the inclusion of length data and separately for males (depicted as squares) and females (depicted as circles). The lefthand panels depict the training datasets and righthand panels the testing datasets. Dashed lines indicate lines of 1:1 agreement between predicted and consensus ages. Solid lines represent linear regression fits to the data. The coefficient of determination (R^2) and mean absolute error (MAE) reported in years are displayed on panels.

Figure 5. Epigenetic age predictions versus consensus ages for the multi-tissue epigenetic clock. Panel A depicts the training dataset and panel B depicts the testing dataset. Dashed lines indicate lines of 1:1 agreement between predicted and consensus ages. Solid lines represent linear regression fits to the data. The coefficient of determination (R^2) and mean absolute error (MAE) reported in years are displayed on panels, with 'FC' indicating fin clips and 'MU' indicating muscle tissue.

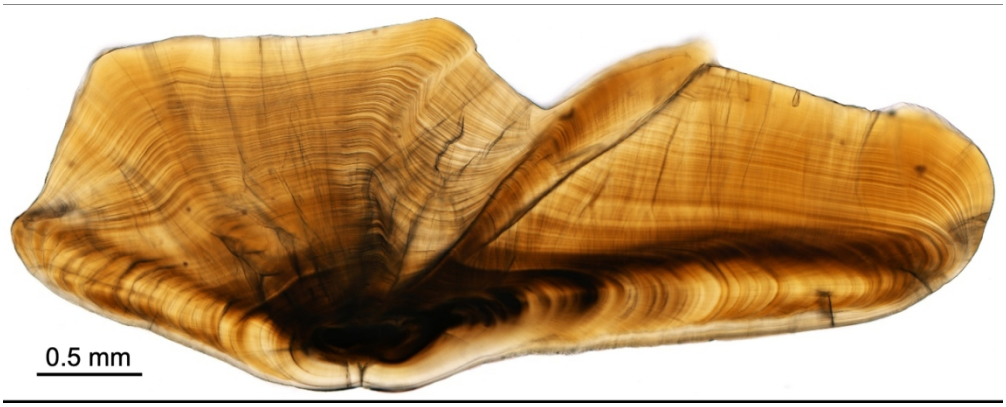

Figure 1. Digital image of a sagittal otolith section viewed with transmitted light from a 352 mm total length female blackbelly rosefish (N1307) with a consensus age estimate of 60 years.

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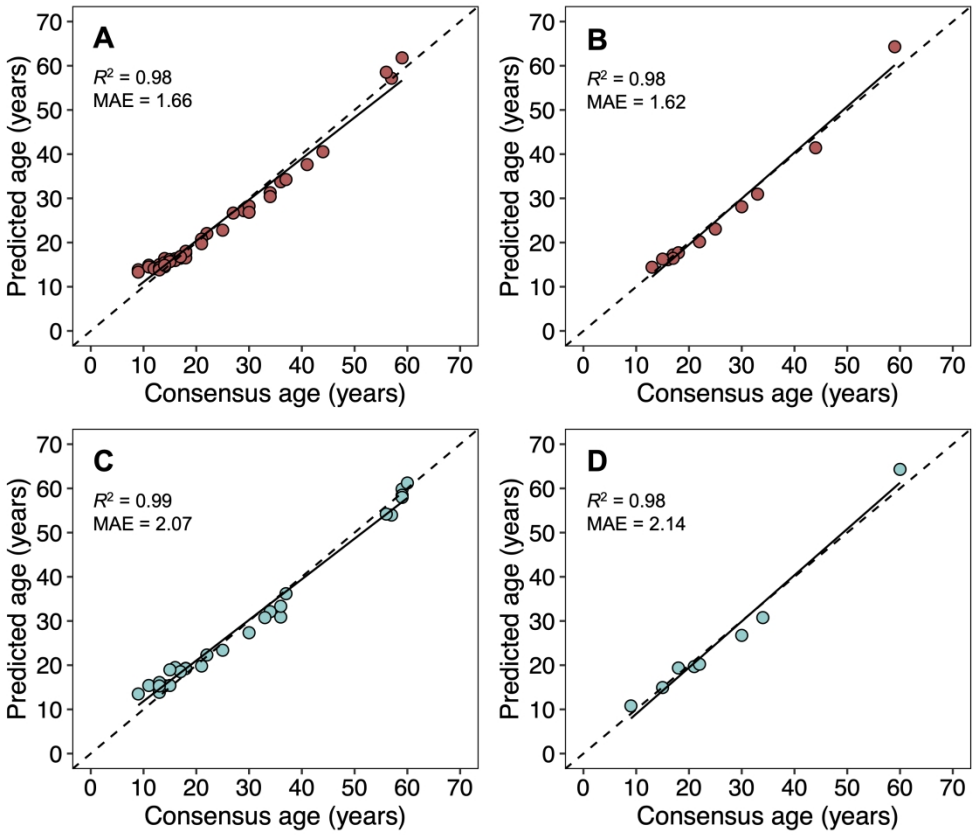

Figure 2. Epigenetic age predictions versus consensus ages for epigenetic clocks developed using fin clip samples (A,B; red circles) and muscle tissue samples (C,D; blue circles). The lefthand panels depict the training datasets and righthand panels the testing datasets. Dashed lines indicate lines of 1:1 agreement between predicted and consensus ages. Solid lines represent linear regression fits to the data. The coefficient of determination (R^2) and mean absolute error (MAE) reported in years are displayed on panels.

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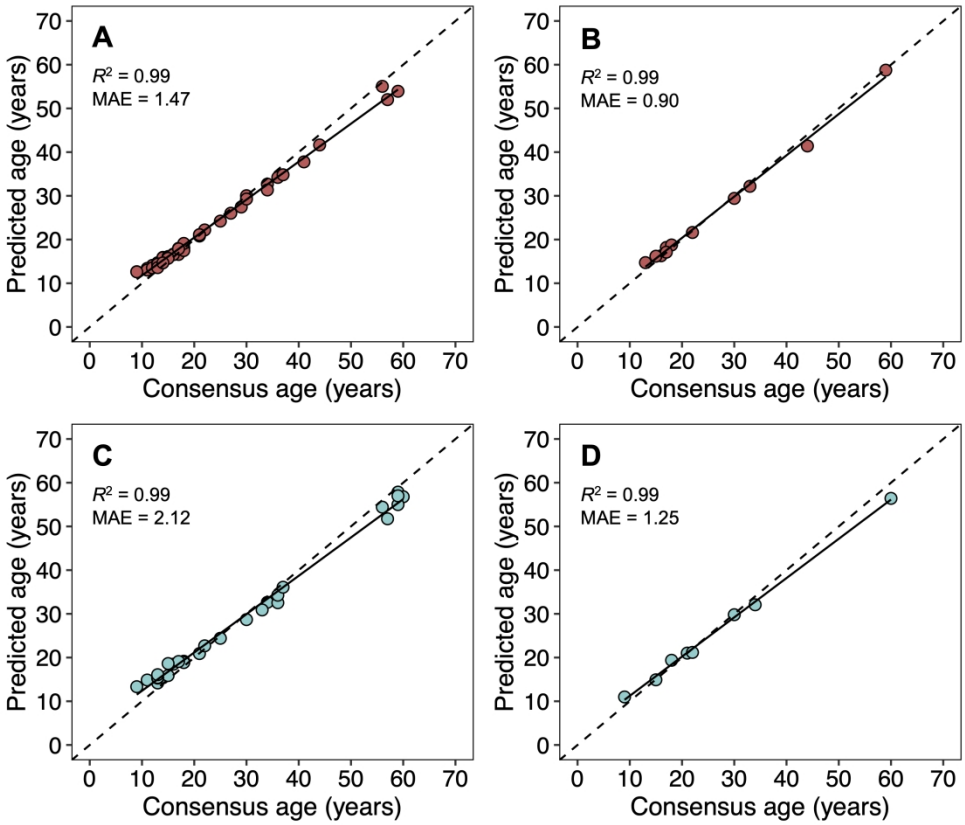

Figure 3. Epigenetic age predictions versus consensus ages for epigenetic clocks developed using fin clip samples (A,B; red circles) and muscle tissue samples (C,D; blue circles) with the inclusion of length data. The lefthand panels depict the training datasets and righthand panels the testing datasets. Dashed lines indicate lines of 1:1 agreement between predicted and consensus ages. Solid lines represent linear regression fits to the data. The coefficient of determination (R^2) and mean absolute error (MAE) reported in years are displayed on panels.

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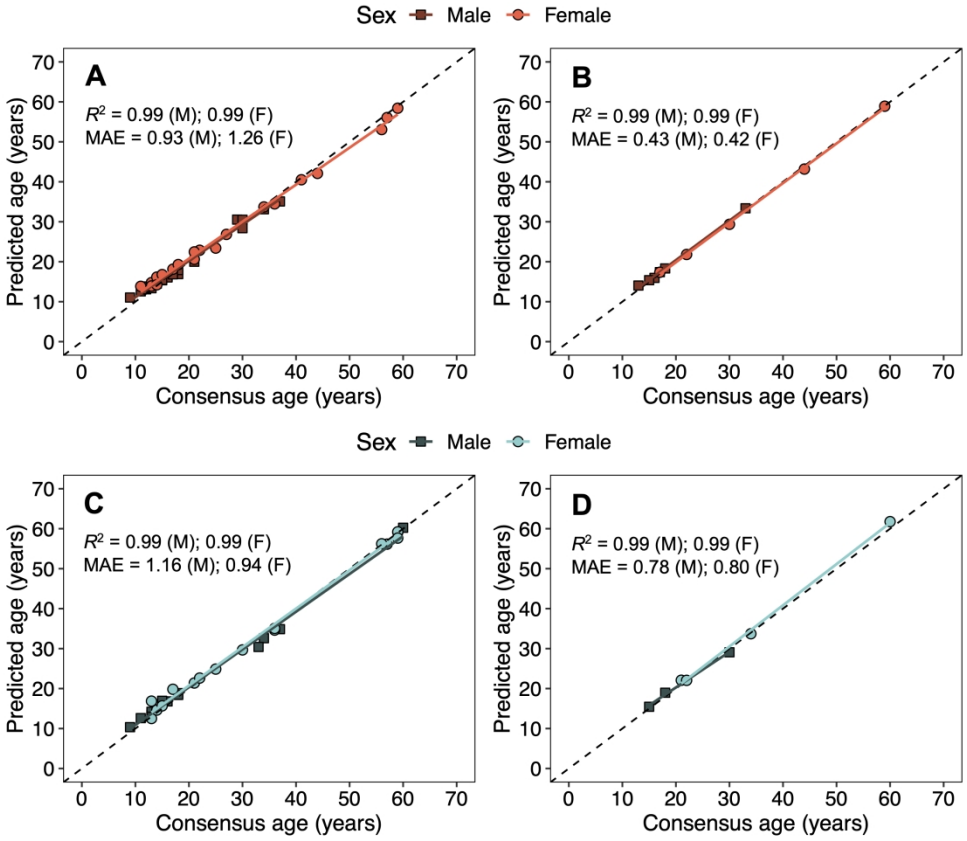

Figure 4. Epigenetic age predictions versus consensus ages for epigenetic clocks developed using fin clip samples (A,B; red) and muscle tissue samples (C,D; blue) with the inclusion of length data and separately for males (depicted as squares) and females (depicted as circles). The lefthand panels depict the training datasets and righthand panels the testing datasets. Dashed lines indicate lines of 1:1 agreement between predicted and consensus ages. Solid lines represent linear regression fits to the data. The coefficient of determination (R^2) and mean absolute error (MAE) reported in years are displayed on panels.

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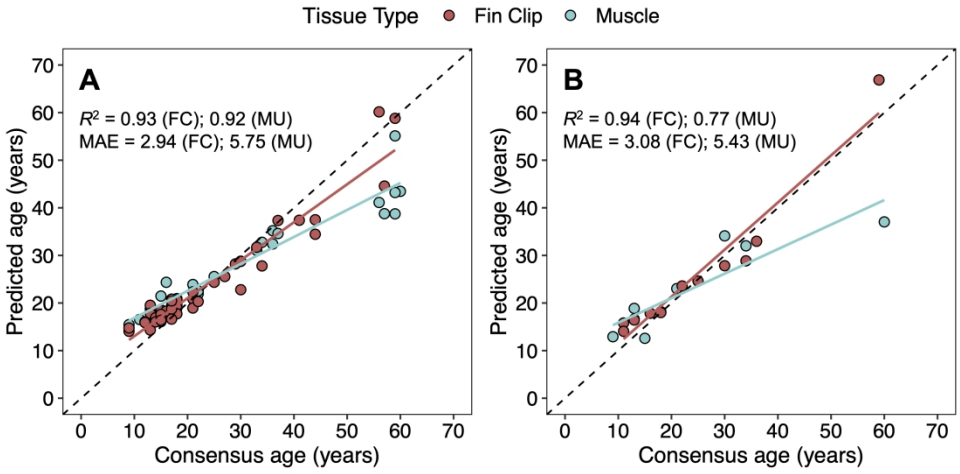

Figure 5. Epigenetic age predictions versus consensus ages for the multi-tissue epigenetic clock. Panel A depicts the training dataset and panel B depicts the testing dataset. Dashed lines indicate lines of 1:1 agreement between predicted and consensus ages. Solid lines represent linear regression fits to the data. The coefficient of determination (R^2) and mean absolute error (MAE) reported in years are displayed on panels, with 'FC' indicating fin clips and 'MU' indicating muscle tissue.

1184x599mm (72 x 72 DPI)